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Effect of Ibuprofen Dose on Platelet Aggregation and Coagulation in Blood Samples From Pigs

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ABSTRACT Introduction: Ibuprofen is commonly used by Soldiers in the deployed environment. This study investigated its dose–effects on in vitro coagulation. Methods: Blood samples were collected from 4 normal healthy pigs and were processed to make platelet-adjusted ($100 \times 10^3/\mu L$) blood samples. Ibuprofen was added to the samples at doses of 0 μg/mL (control), recommended oral dose (163 μg/mL, 1×), 2×, 4×, 8×, 10×, 12×, 16×, and 20×. Arachidonic acid or collagen-stimulated platelet aggregation was assessed at 15 minutes after the addition of ibuprofen. Coagulation was assessed with measurements of prothrombin time (PT) and activated partial thromboplastin time (aPTT), and thrombelastography by Rotem. Results: A robust inhibition of ibuprofen on arachidonic acid-induced platelet aggregation was observed at all doses tested. Collagen-stimulated platelet aggregation was inhibited to 71% ± 5% and 10% ± 5% of the control values at ibuprofen doses of 4× and 20×, respectively (both $p < 0.05$). No changes were observed in PT at any dose, but aPTT was prolonged at dose of 16× and 20×. Rotem measurements of coagulation time, clot formation time, maximum clot firmness, and A10 were compromised at dose 16× and 20× (all $p < 0.05$). Conclusion: Ibuprofen inhibited platelet aggregation at recommended doses, but did not compromise aPTT or coagulation profile until at 16 times the recommended doses and higher. Further effort is needed to clarify whether there are different dose–responses between human and pig blood samples in trauma situations.

INTRODUCTION

Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic properties. It was introduced as a prescription drug in the United States in 1974 and became over-the-counter medicine in 1984. When used at recommended doses (400 mg–800 mg), ibuprofen is considered a safe and effective drug for treatment of pain, inflammation, dysmenorrhea, and fever. However, its widespread availability over the counter can increase risk for its misuse.[1,2] Consequently, the toxic and adverse effects from overdose have been documented, including coma,[1,3–5] seizures,[1] metabolic acidosis,[6] liver injury, gastrointestinal disturbances,[4,5] acute renal failure,[7] thrombocytopenia,[4] and death.[1,8] Previous reports have indicated that ibuprofen can reduce platelet aggregation,[9–11] but its effects on hemostasis are unclear.

In the military, recent reports have revealed previously unrecognized overuse of NSAIDs due to self-treatment of injuries sustained in combat environments.[12] A survey of Soldiers at a forward operating base showed the following pattern of NSAID use: 52% with daily use, 40% with once or twice weekly use, and only 8% with no use.[12] A report from the Military Health System showed that NSAID overdose increased 40% annually from 2004 to 2008 in active duty service members, with junior enlisted 6 times more likely to overdose than officers.[13] Thus, it is imperative to investigate whether overuse of NSAIDs will predispose Soldiers put in harm's way to increased bleeding risk on the battlefield.

Swine are often the animal of choice to investigate the pathophysiology of traumatic hemorrhagic shock and recognize new hemorrhage control and resuscitation treatments for such injuries. Based on the above reports of common ibuprofen use in the military, studies of effects of ibuprofen on hemostasis mechanisms in swine models seem warranted. As a starting point, this study investigated dose–response effects on coagulation function and platelet aggregation in swine blood.

MATERIALS AND METHODS

Pig Blood Collection and Processing

Pig blood was obtained from 4 normal healthy swine being used in another study approved by the Institutional Animal Care and Use Committee of the U.S. Army Institute of Surgical Research, Fort Sam Houston, Texas. This study has been conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals.

Native whole blood (NWB) samples from pig were withdrawn via venous puncture. Blood samples were drawn into citrate tubes containing 3.2% Na Citrate (Becton-Dickenson, Franklin Lakes, New Jersey) and allowed to incubate for 15 minutes at room temperature. An aliquot of the collected NWB was used for measurements of blood gas (the Omni-9 Blood Gas Analyzer, AVL, Montpellier, France) and blood

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cell counts (ABX Pentra 120 Hematology Analyzer, ABX Diagnostics, Irvine, California), including platelet counts and hematocrit. The remaining blood samples were divided into two portions: one was reserved as citrated whole blood samples, and the other was used to make platelet-adjusted whole blood (PAWB) samples, following procedures developed at our Institute.[14] Because platelet counts in pig blood may vary from 120 to 720 × 10^3/μL, and impedance aggregometry results are influenced by platelet counts,[15] a standardized platelet count was selected so as not to mask any potential effects of ibuprofen on coagulation function.[14] Briefly, citrated whole blood samples were centrifuged at 2,000× *g* for 15 minutes to separate platelet-poor plasma (top layer), the buffy coat (middle layer, containing platelets), and red cells (bottom layer). Platelet-poor plasma was first collected via aspiration from the top clear layer. Upon removal of the buffy coat layer under platelet-poor plasma, red blood cells were collected. By adding platelet-poor plasma to collected red blood cells to obtain the hematocrit level identical to that of citrated whole blood samples, a stock of platelet-poor whole blood was made. Afterward, citrated whole blood samples and platelet-poor whole blood samples were mixed in appropriate amounts to obtain a platelet count of 100 × 10^3/μL blood sample, referred to as PAWB samples. Since a platelet level of 100 × 10^3/μL was considered the critical level for platelet transfusion in trauma patients, this level was selected to assess the dose–response effects of ibuprofen in this study. The engineered PAWB was then divided into 8 tubes to test the dose–responses of ibuprofen. The entire study was repeated in 4 separated experiments from blood samples drawn from 4 healthy pigs.

Dosing of Ibuprofen

The FDA-approved standard dose for ibuprofen is up to 800 mg every 6 hours. Assuming an average person of 70 kg has blood volume of 70 mL/kg and with pharmacokinetic data indicating from area under the curve that an oral dose can be nearly 100% absorbed,[16] the estimated plasma concentration from the highest recommended dose would be 163 μg/mL. In the dose set of 8 tubes containing PAWB samples, ibuprofen (Caldolor, IV solution, 100 mg ibuprofen/mL, 78 mg arginine/mL, Nashville, Tennessee) was added at the doses of 0 μg/mL (control), 163 μg/mL (the recommended oral dose, referred to as 1×), 4×, 8×, 10×, 12×, 16×, and 20×, respectively. Buffered blood bank saline (Isotonic solution 0.85% w/v, Thermo Scientific, Waltham, Massachusetts) was used for volume matching among the 8 tubes.

Fifteen minutes after the completion of dosing and volume matching, the dosed blood sample in each tube was aliquoted for 3 measurements: platelet aggregation using the Chrono-Log 700 aggregometer (Chrono-Log, Havertown, Pennsylvania); PT and aPTT by STart (Diagnostica Stago, Rue des Freres Chausson, France); and thrombelastogram by Rotem (TEM, Munich, Germany). All measurements were made at 37°C.

Platelet Aggregation

Platelet impedance aggregometry was assessed in PAWB samples using a Chrono-Log 700 aggregometer. At 15 minutes after the addition of ibuprofen, the aggregation was stimulated with either collagen (2 μg/mL) or arachidonic acid (0.5 mM) as agonists. The area under the curve was used to compare platelet aggregation.

Thrombelastrogram Rotem

Changes in the coagulation profile from PAWB were measured using Rotem. A volume of 300 μL of dosed blood samples was added to the measurement cup followed by addition of Extem reagent, containing recombinant tissue factor and phospholipids, heparin inhibitor, and preservatives. From the tracing of clotting curve, the following parameters were generated to represent coagulation profiles, coagulation time (CT, the time from test start to an amplitude of 2 mm); clot formation time (CFT, the time between 2-mm amplitude and 20-mm amplitude); α-angle (angle between the baseline and a tangent to the clotting curve through the 2-mm point to represent the rate of clot formation); maximum clot firmness (MCF, the maximum amplitude reached during the test); and A_{10} (clot firmness at 10 minutes after CT).

Statistical Analysis

Data were expressed as means ± standard error of the mean from 4 separate experiments and analyzed using SAS statistical software (SAS Institute, Cary, North Carolina). A one-way analysis of variance using a Dunnett adjustment was used to compare the changes to the control (0 dose). The statistically significant level was set at $p < 0.05$.

RESULTS

Blood Characteristics

The characteristics of NWB and PAWB samples are listed in Table I. As designed, platelet count in PAWB was reduced to about 100 × 10^3/μL. There was no difference in other measurements between NWB and PAWB samples (Table I).

Platelet Aggregation

Platelet aggregation was assessed in PAWB using arachidonic acid or collagen as the agonist. Compared to control

TABLE I. Complete Blood Count Measurements of Citrate Whole Blood Samples and Processed Platelet-Adjusted Whole Blood Samples From Blood Taken From 4 Normal Healthy Pigs. Data Are Expressed As Means ± SE From 4 Separated Experiments

	RBC (10^3/μL)	Hematocrit (%)	Hemoglobin (g/dL)	Platelet (10^3/μL)	Fibrinogen (mg/dL)
NWB	5.3 ± 0.2	26.9 ± 0.9	9.0 ± 0.2	336 ± 24	264 ± 12
PAWB	5.2 ± 0.3	26.8 ± 1.7	8.9 ± 0.5	93 ± 7*	240 ± 16

*$p < 0.05$ platelet-adjusted whole blood (PAWB) vs. native whole blood (NWB) samples.

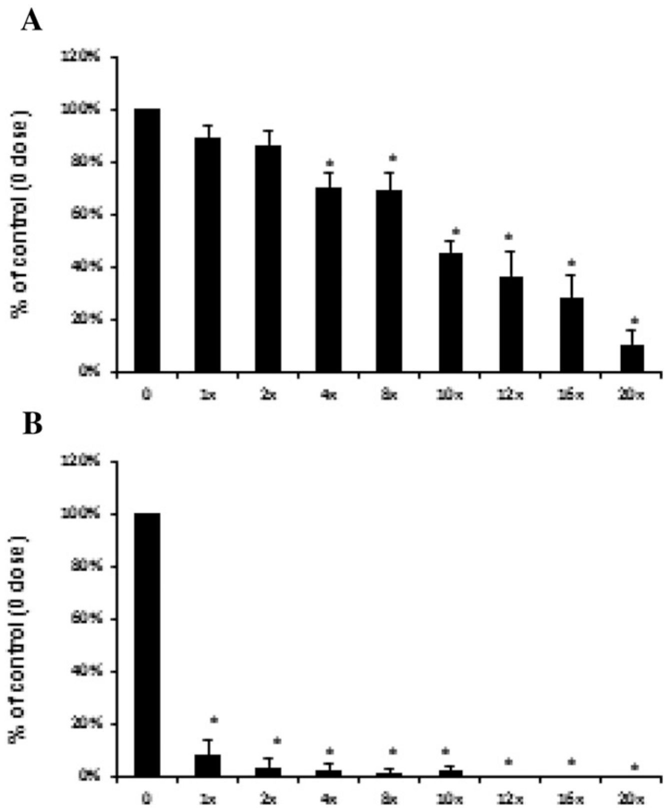

FIGURE 1. (A) Dose–effects of Ibuprofen on collagen-induced and (B) arachidonic acid-induced platelet aggregation in platelet-adjusted pig blood samples. Data are expressed as means ± SE from 4 separated experiments.

(0 dose), ibuprofen inhibited collagen-induced platelet aggregation starting at the 4× dose (Fig. 1). At 4× and 20× of ibuprofen, collagen-induced platelet aggregation was reduced to 71% ± 5% and 10% ± 5% of the control values, respectively (both $p < 0.05$; Fig. 1). A robust inhibition of iburprofen on arachidonic acid-induced platelet aggregation was observed at all doses tested. At 1× and 2× doses of ibuprofen, arachidonic acid-induced platelet aggregation reduced to 8% ± 6% and 4% ± 3% of the control value, respectively (both $p < 0.05$, Fig. 1).

PT, aPTT, and Thromboelastrogram Measurements

The effects of ibuprofen on PT and aPTT were assessed in PAWB samples. At all the doses tested, PT did not change (Table II). However, aPTT was significantly prolonged at doses of 16× and above. Rotem Extem measurements were made in PAWB and shown in Figure 2. The CT and CFT

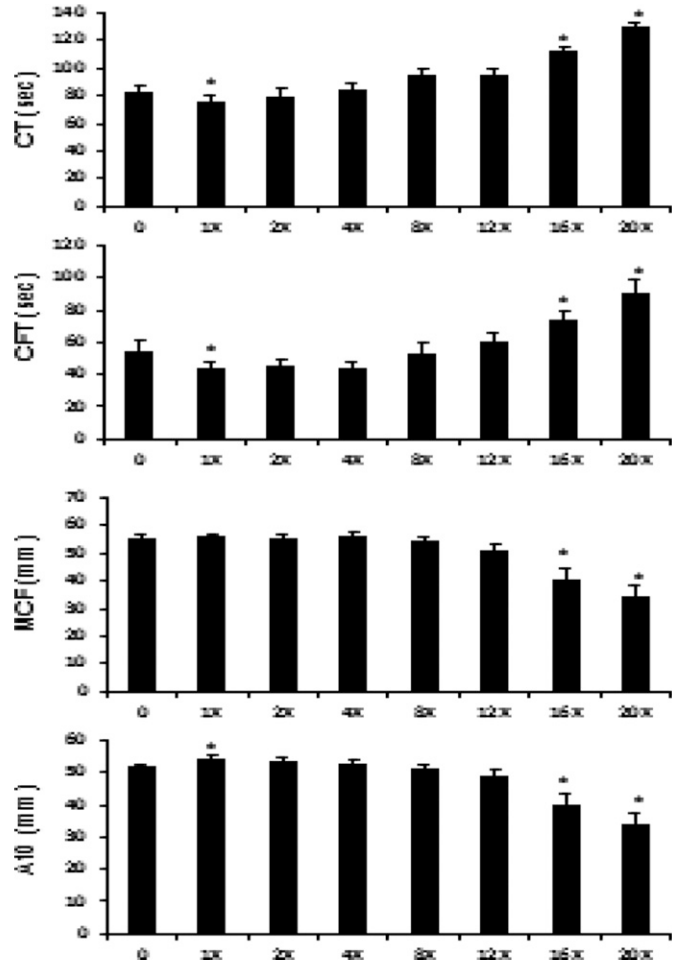

FIGURE 2. Dose–effects of Ibuprofen on Rotem Extem measurements in platelet-adjusted pig blood samples. Data are expressed as means ± SE from 4 separated experiments.

were slightly shorter at 2×, but prolonged at doses 16× and 20× compared to controls. MCF and A_{10} were decreased at doses 16× and 20×, but A_{10} was significantly higher than controls at a dose of 2× (Fig. 2).

DISCUSSION

Hemorrhage is the leading cause of death from potentially survivable injury on the battlefield and a major cause of death in civilian trauma.[17,18] One of the most detrimental consequences of hemorrhage is the development of coagulopathy, increasing mortality 4 times of patients with similar injury severity scores, but without coagulopathy.[19] Damage control

TABLE II. Dose–Effects of Ibuprofen on Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT) From Platelet-Adjusted Pig Blood Samples. Data Are Expressed As Means ± SE From 4 Separated Experiments

	0	1×	2×	4×	8×	12×	16×	20×
PT (Seconds)	15 ± 1	15 ± 1	14 ± 1	15 ± 0	15 ± 1	14 ± 1	14 ± 1	14 ± 1
aPTT (Seconds)	20 ± 2	20 ± 2	20 ± 1	21 ± 1	23 ± 2	25 ± 2	27 ± 2*	31 ± 3*

*$p < 0.05$ compared to control (0 dose) values.

resuscitation (DCR) emphasizes the concept of preventing development or progression of coagulopathy by using blood component therapy and hypotensive resuscitation. Recent reports from military systems have revealed an unrecognized risk factor for coagulopathy: overuse of NSAIDs and acetaminophen.[12,13] The impact of this overuse on DCR is unclear and has not been included in DCR studies. This study was undertaken to assess whether ibuprofen can adversely affect hemostasis. Our data showed that ibuprofen in vitro inhibited platelet aggregation and prolonged aPTT, suggesting that the acute overuse of these drugs may predispose Soldiers to the risk of a self-induced bleeding diathesis, which might potentially impact their survival if they become severely injured.

Primary hemostasis involves a series of complex interactions initiated by platelet contact with damaged subendothelium, initial adhesion, activation, aggregation, degranulation, recruitment of additional platelets, and support of thrombin generation, leading to hemostatic plug formation.[20] Optimal platelet recruitment and subsequent activation during this process is dependent on the synthesis of thromboxane A_2 ($T \times A_2$) from prostaglandin H_2, which is generated from arachidonic acid by cyclooxygenase (COX-1). The antiplatelet effects of various NSAIDs are manifested as impaired platelet aggregation through inhibition of COX-1 activity and reduction of $T \times A2$ synthesis.[20] In the current study, we observed significant inhibition of arachidonic acid-induced platelet aggregation by ibuprofen, starting at its recommended therapeutic dose. Collagen-induced platelet aggregation was also inhibited by ibuprofen, starting at the 4× recommended doses. Since previous reports have shown that ibuprofen inhibits COX-1 activity in a dose-dependent pattern,[10,21] the observed inhibition of collagen-induced platelet aggregation in this study is possibly due to ibuprofen inhibition on COX-1 activities, followed by reduction of $T \times A_2$. Considering the long turnover time of platelets in humans (5–9 days),[22] the marked inhibition of platelet functions by ibuprofen observed in this study supports the notion of using platelet transfusion or products that compensate for reduced platelet function in trauma patients with acute drug overuse and bleeding complications.

The PT test reflects the enzymatic activation of the extrinsic system, including the activation of factors II, VII, and X, whereas the aPTT test represents enzymatic reactions of the intrinsic system, including the activation of factors II, IX, X, XI, and XII. Routine clinical PT and aPTT tests are performed in plasma samples. In this study, PT and aPTT were measured in whole blood samples, which included contributions of cellular components, such as red cells and platelets. Our data showed that ibuprofen did not cause any change in PT at all the doses tested. In contrast, aPTT was prolonged by ibuprofen at the dose of 16× and higher. Our in vitro data are consistent with previous in vivo results. In rabbits and dogs with ibuprofen administration, the primary hemostatic plug formation time and total hemostatic plug formation time from a laser-induced injury in the ear chamber were increased twice (in rabbits) or 5 times (in dogs) of the recommended human doses.[23] In normal healthy volunteers and patients, prolonged bleeding time was observed with 300 mg ibuprofen administration.[9] In addition, the significant changes in aPTT and lack of changes in PT by ibuprofen observed in this study may suggest that ibuprofen has differential effects on the intrinsic and extrinsic pathways of coagulation. Further effort is needed to provide confirmative and mechanistic explanations.

Despite significant inhibition of platelet aggregation at the high end of recommended doses, changes in clotting time and clot strength from Rotem measurements were observed at 16 times of recommended doses. The causes for the differences of the effective doses are unclear. It is possible that at lower ibuprofen doses, the in vitro changes in responsiveness to arachidonic acid, a weak platelet agonist, were masked by large amounts of thrombin generated in response to the activators used for the whole blood PT, aPTT, and Rotem assays. The prolonged whole blood aPTT, prolonged CFT, and reduced MCF observed at high ibuprofen doses may have resulted from a profound platelet inhibition, potentially slowing the activation of platelets to support thrombin generation and/or to participate in fibrinogen cross-linking. The PT, aPTT, and Rotem assays would not be expected to be sensitive to changes in collagen responsiveness because collagen is absent from these assay systems. In this study, we did not perform a Fibtem test on the Rotem or platelet mapping assays on other thromboelastography devices, which might have provided a better picture of platelet dysfunction at lower doses.

It is not clear how the observed effects of ibuprofen on platelet function in pigs will be related to ibuprofen effects on human platelets. We recognize this as a limitation of our study. We are currently performing experiments to compare the dose–effects of ibuprofen in human blood samples to those in pig blood samples. However, porcine models have been extremely valuable in the study of treatments for hemorrhagic shock and polytrauma. This preliminary, in vitro study of coagulation and platelet aggregation in swine blood is warranted in preparation for future studies in trauma models, and to help determine the translatability of these experimental studies to human trauma patients.

It is worth emphasizing that coagulation changes from ibuprofen in this study did not include systemic effects, which, therefore, might underestimate the adverse effects of the drug. For example, all NSAIDs may be considered potentially hepatic toxic, especially at high concentrations,[24,25] and ibuprofen is not an exception. Mild effects on liver function and hepatic toxicity related to ibuprofen have been reported, including a case with 1,200 mg/day for 28 days.[26] Compromised hepatic function has been considered closely related to coagulation disturbances.[27,28] When liver function is impaired, synthesis of coagulation factors is inhibited and coagulation factors are more rapidly depleted. Consistently, clinical case reports have shown that ibuprofen overdose is associated with prolonged bleeding times.[29] Thus, the in vitro

results from the present study, which only show the direct effects of ibuprofen, may possibly underestimate the in vivo effects of ibuprofen if ibuprofen metabolites have greater effects on coagulation than the parent drug, but this is not known and would be more of an issue with chronic misuse of the drug.

Aspirin is another widely used NSAID. Despite similar anti-inflammatory, analgesic, and antipyretic effects, different profiles of actions have been observed in Aspirin and ibuprofen. At equivalent effective doses of ibuprofen (2,400 mg/day, equivalent to $3\times$ in this study) and aspirin (3,900 mg/day), liver function and platelet aggregation are more adversely affected by aspirin.[30,31] Further, compromised platelet aggregation by ibuprofen returned to normal within 24 hours after cessation of the drug, but it took a week to return to normal after aspirin because of its irreversible effects on platelets.[31] However, despite a better safety profile compared with that of aspirin, ibuprofen should still be used with caution because of its adverse effects on hemostasis. Meloxicam, a new NSAID with FDA approval in 2006, is recently included of the pill pack in the U.S. military. Meloxicam is considered to have less interference with platelet function and fewer gastrointestinal side effects, although confirmative evidence remains to be found. Our ongoing effort is comparing the effects of different NSAIDs on hemostasis.

Recently, it has been demonstrated that the acute coagulopathy of trauma includes a component of reduced platelet function, separate from any medications taken by trauma victims. Kutcher et al[32] reported that 45.5% of critically injured patients had reduced platelet responsiveness to one or more agonists at the time of admission. This raises the possibility that an impairment in platelet function as a result of self-medication with ibuprofen may be additive to the platelet impairment that is directly because of trauma. In theory, this may result in an increased propensity for bleeding, as has been observed in nontrauma patients on dual antiplatelet therapy.[33]

In conclusion, we investigated dose–responses of ibuprofen on hemostasis in pig blood samples in vitro. Ibuprofen inhibited platelet aggregation at near-standard recommended doses, prolonged aPTT starting at 16 times of standard doses, and compromised coagulation profile starting at 16 times the standard dose. Continuing research effort is ongoing to investigate whether there are different dose–responses of ibuprofen between human and pig blood samples in trauma situations.

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